STRUCTURAL ROLE OF AMINO ACIDS 99-110

IN RECOMBINANT HUMAN ERYTHROPOIETIN

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SUMMARY

Erythropoietin is the prime regulator of red blood cell production. Previous studies demonstrated that antipeptide antibodies to amino acids 99-119 and 111-129 bind to two non-overlapping domains and inhibit the hormone's action (Sytkowski, A.J. and Donahue, K.A. (1987) J. Biol. Chem. **262**, 1161-1165). Oligonucleotide-directed mutagenesis now shows that amino acids 99-110 (domain 1) but not 119-129 (domain 2) are important to erythropoietin's structure and function. Mutagenesis of wild type human erythropoietin cDNA was used to produce a series of mutant proteins with sequential deletion of three adjacent amino acids and insertion of the sequence Glu-Phe across the two domains. Transient expression in COS-7 cells revealed 2.0 kb transcripts encoded by all of the cDNAs. Domain 2 mutants exhibited specific biological activities similar to that of the wild type. In contrast, domain 1 mutants were not secreted. In vitro transcription and translation of the domain 1, domain 2 and wild type cDNAs resulted in 23.5 kDa and 32 kDa proteins in the absence or presence of pancreatic microsomes, respectively,

consistent with efficient translation of all of the mutants and equivalent post-translational processing of each protein. The data suggest that mutation within domain 1 results in the intracellular biosynthesis of erythropoietins with altered structure, rendering them subject to rapid degradation. Bioassay of erythropoietins synthesized entirely in vitro demonstrated that domain 1 mutants were inactive, whereas both wild type and domain 2 mutant hormones exhibited biologic activity. The results are consistent with a critical role for amino acids 99-110 in the structure of human erythropoietin.

INTRODUCTION

The glycoprotein hormone erythropoietin regulates the growth and differentiation of red blood cell progenitors. Several approaches have been employed to identify those features of the protein that are relevant to its structure and function. Examination of the homologies among the amino acid sequences of erythropoietin molecules of various species has demonstrated several highly conserved regions (McDonald et al., 1986). The role of oligosaccharide chains in the hormone's biological activity is controversial (Dordal et al., 1985; Lai et al., 1986; Sasaki et al., 1987; Wojchowski et al., 1987; Dubé et al., 1988; Takeuchi et al., 1988; Tsuda et al., 1988; Takeuchi et al., 1989; Tsuda et al., 1990; Takeuchi et al., 1990, Sytkowski et al., 1991).

We have shown previously, using a monoclonal antipeptide antibody, that the amino terminus of erythropoietin is not relevant to biological activity (Sytkowski & Fisher, 1985), a conclusion that has been confirmed independently (Wognum et al., 1988). Subsequently, we used antipeptide antibodies to several

hydrophilic domains of the molecule and demonstrated that antibodies to amino acids 99-119 and 111-129 block the hormone's biological activity, apparently by binding to two distinct non-overlapping domains (99-110 and 120-129) (Sytkowski & Donahue, 1987). Based upon these studies, we hypothesized that amino acids 99-129 were important in the formation of a functional region involved in receptor recognition, either through forming a necessary component of the protein's tertiary structure or through direct participation in receptor binding, or both. In contrast, amino acid sequences 1-20, 40-59, 80-99, and 131-150 of erythropoietin do not appear to participate in receptor recognition or activation (Sytkowski & Fisher, 1985; Sytkowski & Donahue, 1987).

Preliminary experiments suggested that alterations in localized secondary structure within the 99-129 region resulted in inactivation of erythropoietin. This is especially relevant since the binding of antibody presumably also changes such localized structure significantly. Therefore, we have sought to

examine the possibility of a structural role for amino acids 99-129 through oligonucleotide-directed mutagenesis of the 99-110 and 120-129 domains. The data indicate that amino acids 99-110 play a critical role in establishing the biologically active conformation of human erythropoietin.

MATERIALS AND METHODS

Cell culture and transfection

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO). Transient expression of cDNAs was performed using a DEAE-Dextran protocol modified by 0.1 mM chloroquine treatment (Sussman & Milman, 1984; Ausubel et al., 1989). Three days before the transfection, COS-7 cells were plated at 2 X 10^5 per 10 cm tissue culture dish. 4 ug of DNA were used in each transfection. Medium was collected 3 days after transfection and assayed for erythropoietin activity and protein.

Erythropoietin assays

The biological activity of erythropoietin samples was determined by in vitro bioassay (Krystal, 1983). Erythropoietins synthesized by in vitro translation were dialyzed against assay medium before bioassay. Determinations were made in triplicate. Erythropoietin protein was determined in duplicate by competitive radioimmunoassay calibrated against recombinant human erythropoietin (Incstar).

Oligonucleotide-directed mutagenesis

The cDNA of wild type human erythropoietin was the generous gift of Dr. J.S. Powell (Dubé et al, 1988). A 1.3 kb Eco RI-Eco RI fragment was subcloned from an SP70 vector into a Eco RI cloning site of an M13mp18 vector using standard procedures (Maniatis et al., 1982). Each mutagenesis employed an oligonucleotide of 48 bases which was designed to replace three codons (or, in one case, four codons) of human erythropoietin cDNA (in the region from amino acid 99 to 129) with an Eco RI restriction site (-GAATTC-) encoding Glu-Phe. Oligonucleotides

were synthesized using an Applied Biosystems DNA synthesizer 380A (Foster, CA) and purified through oligonucleotide purification cartridges (OPC^{TM}, Applied Biosystems). Oligonucleotide-directed mutagenesis was performed using the "Muta-Gene M13 *in vitro* Mutagenesis Kit" (Bio-Rad, Richmond, CA) based on a method described by Kunkel (Kunkel et al., 1985). The M13 phage containing the mutated erythropoietin cDNA was then infected into *E. coli* CJ236, the dut,ung double mutant bacterium (Kunkel et al., 1987) from which the single stranded uracil-containing viral DNA was obtained. An oligonucleotide complementary to erythropoietin cDNA with an internal mismatch to the region to be mutated was hybridized to the single stranded uracil-containing viral erythropoietin DNA. A non-uracil-containing complementary strand DNA was synthesized *in vitro* by DNA polymerase. The resulting DNA hybrid was transformed into bacterial cells with active uracil N-glycosylase, resulting in inactivation of the uracil-containing parent DNA. A 1.1 kb Sst I-Bst XI fragment containing the mutated site was then subcloned into the pSV2-Epo^{wt} constructed using standard procedures (Mulligan et al.,

1979; Maniatis et al., 1982).

Total cytoplasmic RNA extraction and Northern blot

Cytoplasmic RNA was prepared using guanidinium isothiocyanate (Chirgwin et al., 1979). Fifty micrograms of total RNA was fractionated electrophoretically on 1.2% agarose containing 5.5% formaldehyde and transferred to GeneScreen Plus (DuPont, MA). The filter was hybridized with a ^{32}P-labeled 1.1 kb Sst I-Bst XI fragment of human erythropoietin (Jacobs et al., 1985). The radiolabeled probes were generated by random-primed labeling (Feinberg & Vogelstein, 1984).

In vitro transcription

Epo^{wt}, Epo^{99}, Epo^{102}, Epo^{105}, Epo^{108}, and Epo^{120} cDNAs were subcloned from the pSV2 vector into the Eco RI cloning site of the pSP70 vector (Promega, Wisconsin) using standard procedures (Maniatis et al., 1982). This pSP70 plasmid contains the bacteriophage SP6 and T7 RNA polymerase promoters in opposite orientations flanking a region of multiple restriction cloning sites. In vitro transcription of erythropoietin mutants was

driven by T7 RNA polymerase (Promega). The orientation of the erythropoietin cDNA insert related to the promoters was confirmed by restriction enzyme mapping. The in vitro transcription reaction was performed using a protocol of Melton (Melton et al., 1984) with slight modification. Erythropoietin cDNA constructs were linearized by Hind III before the transcription reaction. 5 ug of the linear DNA template were added to 45 ul of transcription mixture containing 40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM $CaCl_2$, 10mM dithiothreitol, 1 U/ml RNasin ribonuclease inhibitor (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.5 mM GTP, 0.5 mM GpppG, 40 U T7 RNA polymerase and incubated at 37°C for 60 min. To increase the yield of RNA, an additional 40 U of T7 RNA polymerase were added to the reaction of mixture and incubated for another 60 min at 37°C. To remove the DNA template, 60 units of RQ1 RNase-free DNase (Promega) were added and incubated for 15 min at 37°C. Unincorporated nucleotides were removed by ethanol precipitation.

In vitro translation and processing

In vitro translation of Epo^{wt}, Epo^{99}, Epo^{102}, Epo^{105}, Epo^{108}, and Epo^{120} was performed using a rabbit reticulocyte translation system (Promega, Wisconsin). mRNA (0.5 ug) was added to the translation mixture (25 µl total volume) containing 17.5 µl nuclease treated rabbit reticulocyte lysate, 0.01 mM amino acid mixture (minus cysteine), 20 µCi [^{35}S]cysteine (Dupont/NEN, 1027 Ci/mmol) or 0.01 mM unlabeled cysteine, 1 U/ml RNasin ribonuclease inhibitor (Promega) and incubated at 30°C for 60 min. Co-translational and initial post-translational processing of protein, such as signal peptide cleavage and core glycosylation, were examined by addition of canine pancreatic microsomal vesicles (Promega, Wisconsin) during the in vitro translation reaction (Walter & Blobel, 1983). The translation products were visualized by immunoprecipitating [^{35}S]cysteine-labeled translation products with a rabbit polyclonal anti-erythropoietin antibody followed by electrophoresis on 12% SDS polyacrylamide gels (Laemmli, 1970) and autoradiography.

Immunoprecipitation of erythropoietin

Wild type and mutant erythropoietins synthesized by in vitro translation were diluted 10-fold with NET buffer (150 mM NaCl; 50 mM Tris-HCl, pH = 7.5; 5 mM EDTA; 1.5% NP-40 and 2.5 mg/ml bovine serum albumin). After overnight incubation at 4°C with a rabbit polyclonal anti-erythropoietin antibody, an equal volume of formalin-fixed Staphylococcus aureus (Cowan I strain; Enzyme Center) (Sue & Sytkowski, 1983) prepared in TDB buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5% w/v bovine serum albumin, 0.2% sodium azide, 0.25 mM sodium phosphate, 150 mM NaCl, pH 7.5) was added to the samples, and the suspension was incubated for 30 minutes at room temperature. The cells were pelleted by centrifugation and washed twice with TDB buffer. Immunoprecipitates were dissociated in 30 µl of 2 X SDS electrophoresis sample buffer, and the supernatants were analyzed on 12% SDS polyacrylamide gels. The gels were dried at 80°C under vacuum and autoradiographed.

RESULTS

A series of mutant erythropoietin cDNAs was prepared by oligonucleotide-directed mutagenesis of the wild type cDNA. The mutation was designed to remove groups of three (or four) amino acids sequentially and to replace them with two amino acids (Glu-Phe), thus perturbing localized secondary structure systematically. By removal of nine bases and introduction of the sequence GAATTC, a new Eco RI restriction site was introduced into the cDNA at the site of the mutation, thereby allowing confirmation of the mutation by restriction analysis. The mutations (Table 1) were designed to move from 5′ to 3′ across two domains: domain 1, amino acids 99-110, and domain 2, amino acids 120-129. We based this analysis on our previous antibody studies which indicated that antibodies to each of these two domains blocked erythropoietin's biological activity (Sytkowski & Donahue, 1987). Each of the mutant and wild type cDNAs was then subcloned into the expression plasmid pSV2 (Figure 1). Plasmid was isolated and transfected into COS-7 cells transiently.

Table 1

Figure 1

Northern blot analysis of COS-7 cells transfected with either wild type or mutant cDNA demonstrated that each cDNA was transcribed, resulting in comparable steady state levels of transcript of equivalent size, 2.0 kb (Figure 2). The supernatant culture medium of each transfected culture was subjected to bioassay and radioimmunoassay for erythropoietin (Table 2). The wild type erythropoietin cDNA (Epo^{wt}) was expressed efficiently in these cells. The culture medium contained 2.5 ± 0.1 IU/ml with a calculated specific activity of 210 IU/μg, identical to that obtained for highly purified recombinant human erythropoietin. In contrast, none of the cultures transfected with mutants from domain 1 (Epo^{99}, Epo^{102}, Epo^{105}, Epo^{108}) contained either biologically active or radioimmunoassay detectable erythropoietin. However, all cultures transfected with domain 2 mutants (Epo^{120}, Epo^{123}, Epo^{126}) contained biologically active erythropoietin. The specific activities of Epo^{120} and Epo^{123} were slightly higher than Epo^{wt} (270 vs 210 IU/μg), whereas the specific activity of Epo^{126}, from which four amino acids were deleted, was somewhat

lower (140 IU/μg). No erythropoietin protein was detectable in the cell pellets from the domain 1 mutants. The data strongly suggest that domain 2 is probably not directly involved in receptor binding.

The absence of detectable erythropoietin protein expressed from the domain 1 mutants, despite similar steady state levels of transcript (Figure 2), implies that mutations in domain 1 either resulted in mRNAs that could not be translated efficiently, or led to structural changes in the erythropoietin protein. Such changes in protein structure could result in

1) deficient post-translational modification/processing,

2) increased susceptibility to cellular proteases and/or

3) a block in the secretory process.

In order to assess the ability of mRNA from domain 1 mutants to be translated, we prepared mRNAs by in vitro transcription and then examined their activity by in vitro translation. Each of the domain 1 mutant cDNAs (Epo^{99}, Epo^{102}, Epo^{105}, Epo^{108}) as well as domain 2 mutant Epo^{120} and Epo^{wt} cDNA were subcloned into the

pSP70 expression vector (see Materials and Methods). A Northern blot analysis of the in vitro transcription of each cDNA revealed nearly equal synthesis of a 2 kb transcript in all cases (not shown). This transcript was isolated and subjected to in vitro translation. Translation was carried out in the absence or presence of pancreatic microsomes in order to evaluate 1) functionality of each mRNA and 2) capability of each protein to be processed. After in vitro translation in the presence of [^{35}S]cysteine, the erythropoietins were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

Translation of each mRNA in vitro in the absence of pancreatic microsomes resulted in an unprocessed protein of approximately 23.5 kDa, very similar to that predicted for the erythropoietin precursor protein, 21.3 kDa (Jacobs et al., 1985) (Figure 3). Addition of pancreatic microsomes to the translation systems resulted in 32 kDa proteins in all cases, consistent with core glycosylation and, presumably, removal of the leader sequences of the proteins. These results indicate that the mRNAs

Figure 3

of the domain 1 mutants were translated efficiently and that the resultant proteins were equally susceptible to post-translational modification/processing as were the normally active domain 2 mutant Epo^{120} and wild type Epo^{wt}. Thus, we conclude that the absence of secreted erythropoietin from COS-7 cell cultures transfected with the domain 1 mutants (Table 2) and from the cell pellets was most likely due to increased susceptibility of the domain 1 mutant proteins to proteolysis within the cell. This is consistent with an important structural change imparted by the domain 1 mutations.

In order to assess the biologic activity of the domain 1 mutants, we determined the activities of the erythropoietins synthesized and processed *in vitro*. Portions of translation mixtures (Figure 3) were dialyzed and subjected to *in vitro* bioassay and radioimmunoassay (Table 3). Importantly, Epo^{wt} and the domain 2 mutant Epo^{120} were both active when biosynthesized *in vitro*, as expected. In contrast, all four domain 1 mutants were inactive. Virtually identical results were obtained in two additional experiments. The results indicate that the mutations

Table 3

introduced in domain 1 but not in domain 2 inactivate erythropoietin.

DISCUSSION

In this study, we have introduced systematically a 3 amino acid deletion ---> 2 amino acid insertion mutation stepwise across two domains of human erythropoietin. We hypothesized that this mutation would perturb the localized secondary structure thereby permitting an initial assessment of the importance of this region to the protein's structure/function. The data show that domain 1 (amino acids 99-110) is important in the maintenance of a stable, biologically active conformation. The finding that mutagenesis across domain 2 (amino acids 120-129) does not alter erythropoietin's biological activity significantly, although antibodies to this region do block its activity (Sytkowski and Donahue, 1987), indicates that the antibody to domain 2 probably inhibits the hormone by steric hindrance of some other region, possibly the adjacent domain 1. A deletion mutant lacking amino acids 111-119

reportedly exhibits nearly full biologic activity, in agreement with our results (Biossel & Bunn, 1990).

The absence of any x-ray crystallographic data on erythropoietin requires that structural conclusions based upon the information presented here be somewhat speculative. Examination of predicted secondary structure by the method of Garnier (Garnier et al., 1978) reveals a 13 amino acid stretch of extended conformation consisting of amino acids 98-110 of wild type erythropoietin. Computer analysis of the domain 1 mutants Epo^{99}, Epo^{102} and Epo^{105} demonstrates the conversion of this stretch of extended conformation to α helix, joining two other helices on either side and resulting in a helical conformation from amino acids 90-115. However, mutation Epo^{108} does not result in this predicted stretch of α helix but rather is predicted to only modestly perturb the extended conformation, shortening it by 1 amino acid. Yet, Epo^{108} is also inactive. The algorithm of Chou and Fasman (Chou & Fasman, 1977; Chou & Fasman 1979) reveals a prominent high probability ß turn centered

at amino acid 120. Although domain 1 mutations have no effect on this turn, mutation Epo^{120} completely obliterates it and Epo^{123} reduces its probability markedly. However, there is no effect on erythropoietin's activity with these latter two mutations. Thus, initial computer analyses of predicted secondary structure do not fully explain the results of mutagenesis.

Our demonstration of an important structural role for amino acids 99-110 of erythropoietin does not rule out their participation in receptor recognition as well. Indeed, the initial interaction of a hormone with its cognate receptor might be expected to result in further conformational changes of the hormone ligand, thereby stabilizing the hormone-receptor complex and possibly allowing the formation of higher ordered complexes. We anticipate that mutation of individual amino acids coupled with suitable determination of tertiary structure will be necessary to characterize the hormone's receptor binding domain fully.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., & Struhl, K. (1989) in Current Protocols in Molecular Biology pp. 921-926, John Wiley and Son, New York

Biossel, J-P., & Bunn, H.F. (1990) in The Biology of Hematopoiesis, (Dainiak, N., Cronkite, E.P., McCaffrey, R., & Shadduck, R.K., eds) pp. 227-232, Wiley-Liss, New York

Chirgwin, J.J., Pryzbyla, A.E., MacDonald, R.J., & Rutter, W.J. (1979) Biochemistry **18**, 5294-5299.

Chou, P.Y., & Fasman, G.D. (1977) J. Mol. Biol. **115**, 135.

Chou, P.Y., & Fasman, G.D. (1979) Biophys. J. **26**, 367.

Dordal, M.S., Wang, F.F., & Goldwasser, E. (1985) Endocrinology **116**, 2293-2299.

Dubé, S., Fisher, J.W., & Powell, J.S. (1988) J. Biol. Chem. **262**, 17516-17521.

Feinberg, A.P. & Vogelstein, B. (1984) Anal. Biochem. **137**, 266-272.

Garnier, J., Osguthorpe, D.J., & Robson, B. (1978) J. Mol. Biol. **120**, 97.

Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D., Kaufman, R.J., Mufson, A., Seehra, J., Jones, S.S., Hewick, R., Fritsch, E.F., Kawakit, M., Shimizu, T., & Miyake, T. (1985) Nature **313**, 806-810.

Krystal, G. (1983) Exp. Hematol **11**, 649-660.

Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA **82**, 488-492.

Kunkel, T.A., Roberts, J.D., & Zabour, R.A. (1987) Methods Enzymol. **154**, 367-382.

Laemmli, U.K. (1970) Nature **227**, 680-685.

Lai, P.-H., Everett R., Wang, F.-F., Arakawa, T., & Goldwasser, E. (1986) J. Biol. Chem. **261**, 3116-3121.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. **193**, 265-274.

Maniatis, T. Fritsch, E.F., & Sambrook, J. (1982) Cold Spring Harbor Laboratories, Cold Spring Harbor, New York

McDonald, J.D., Lin, F.K., & Goldwasser, E. (1986) Mol. Cell Biol. **6**, 842-848.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., & Green, M.R. (1984) Nucleic Acids Res. **12**, 7035-7056.

Mulligan, R.C., Howard B.H. & Berg, P. (1979) Nature 277, 108-114.

Sasaki, H., Bothner, B., Dell, A., & Fukuda, M. (1987) J. Biol. Chem. 262, 12059-12076.

Sue, J.M., & Sytkowski, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 3651-3655.

Sussman, D.J., & Milman, G. (1984) Mol. Cell Biol. 4, 1641-1645

Sytkowski, A.J., & Donahue, K.A. (1987) J. Biol. Chem. 262, 1161-1165.

Sytkowski, A.J., Feldman, L., Zurbuch, D.J. (1991) Biochem. Biophys. Res. Commun. 176, 698-704.

Sytkowski, A.J., & Fisher, J.W. (1985) J. Biol. Chem. 260, 14727-14731.

Takeuchi, M., Inoue, N., Strickland, T.W., Kubota, M., Wada, M., Shimizu, R., Hoshi, S., Kozutsumi, H., Takasaki, S., & Kobata, A. (1989) Proc. Natl. Acad. Sci. USA 86, 7819-7822.

Table 1. Erythropoietin cDNA mutations and predicted amino acid sequence changes

Each DNA deletion was replaced with -GAATTC- resulting in the introduction of amino acids -Glu-Phe- for the deleted ones.

Construct	DNA codon deletion	Protein sequence position affected	Domain affected	Amino acids deleted
Epo^{wt}	None (wild type)		-	
Epo^{99}	GTC-AGT-GGC	99-101	1	Val_{99}-Ser_{100}-Gly_{101}
Epo^{102}	CTT-CGC-AGC	102-104	1	Leu_{102}-Arg_{103}-Ser_{104}
Epo^{105}	CTC-ACC-ACT	105-107	1	Leu_{105}-Thr_{106}-Thr_{107}
Epo^{108}	CTG-CTT-CGG	108-110	1	Leu_{108}-Leu_{109}-Arg_{110}
Epo^{120}	TCC-CCT-CCA	120-122	2	Ser_{120}-Pro_{121}-Pro_{122}
Epo^{123}	GAT-GCG-GCC	123-125	2	Asp_{123}-Ala_{124}-Ala_{125}
Epo^{126}	TCA-GCT-GCT-CCA	126-129	2	Ser_{126}-Ala_{127}-Ala_{128}-Pro_{129}

Table 2. Erythropoietin biological activity and protein in medium from transfected COS-7 cell cultures

Data expressed as means ± SD of triplicate determination. Activity determined by bioassay, Epo protein by RIA and cell pellet protein by method of Lowry (Lowry et al., 1954). N.D. = none detected.

Construct	Secreted biological activity, IU/ml culture	Specific activity IU/μg Epo	Erythropoietin in cell pellet, ng Epo/mg cell protein
Epo^{wt}	2.5 ± 0.1	210 ± 10	10±1
Epo^{99}	ND	-	ND
Epo^{102}	ND	-	ND
Epo^{105}	ND	-	ND
Epo^{108}	ND	-	ND
Epo^{120}	2.8 ± 0.1	270 ± 15	11±1
Epo^{123}	3.1 ± 0.1	270 ± 15	13±1
Epo^{126}	0.8 ± 0.1	140 ± 25	4±1

Table 3. Biological activity of erythropoietins synthesized in vitro

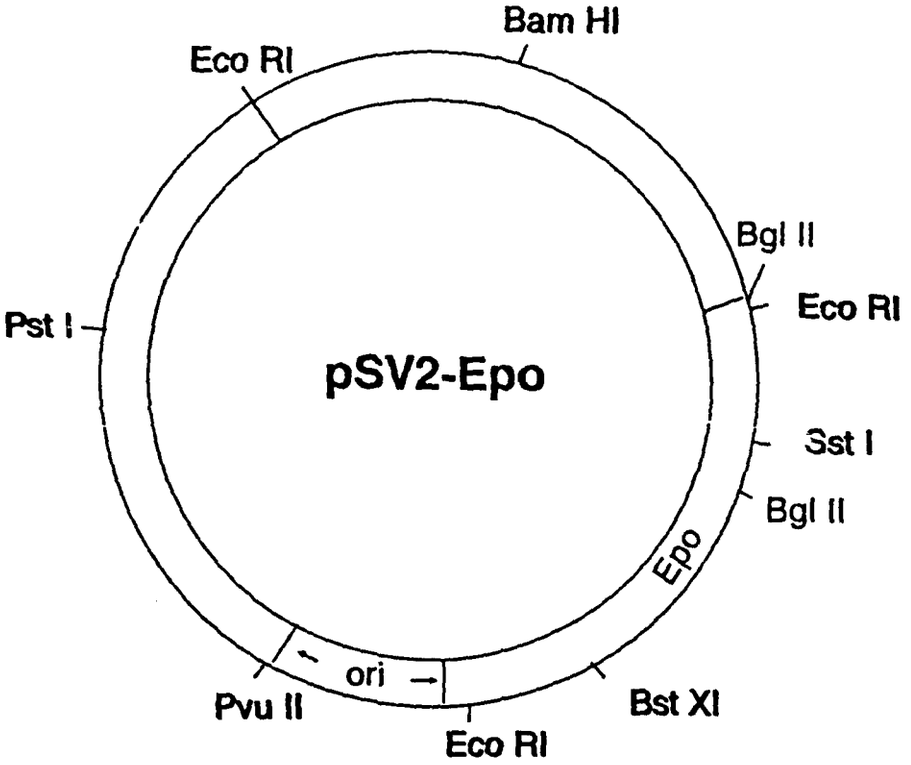
Construct	Biological activity, IU/ml	Erythropoietin protein, ng/ml	Specific activity IU/μg
Epo^{wt}	1.58 ± 0.10	7.8 ± 0.1	200 ± 10
Epo^{99}	ND	8.1 ± 0.2	-
Epo^{102}	ND	7.4 ± 0.1	-
Epo^{105}	ND	7.7 ± 0.1	-
Epo^{108}	ND	7.1 ± 0.2	-
Epo^{120}	2.25 ± 0.05	10.7 ± 0.2	210 ± 15

FIGURE LEGENDS

Figure 1: Structure of expression vector pSV2-Epo. Epo, erythropoietin cDNA insert.

Figure 2: Northern blot analysis of total cellular RNA from transfected COS-7 cell cultures. Filters were probed with a ^{32}P-labeled 1.1 kb Sst I-Bst XI fragment of human erythropoietin. Cells were transfected with: pSV2 = control plasmid without insert; 99,102,105,108,120,123,126 = pSV2 with mutant cDNA inserts (see Table 1); wt = pSV2 with wild type cDNA.

Figure 3: Synthesis and post-translational processing of erythropoietins produced by *in vitro* transcription and translation. mRNAs shown were transcribed *in vitro* and translated in the absence or presence of pancreatic microsomes. Note the 23.5 kDa and 32 kDa proteins synthesized from each mRNA. See Materials and Methods and Results.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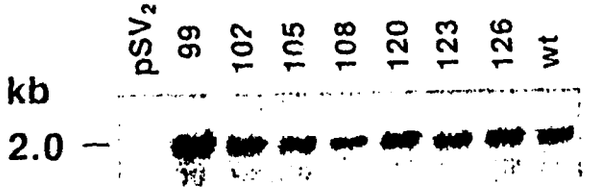
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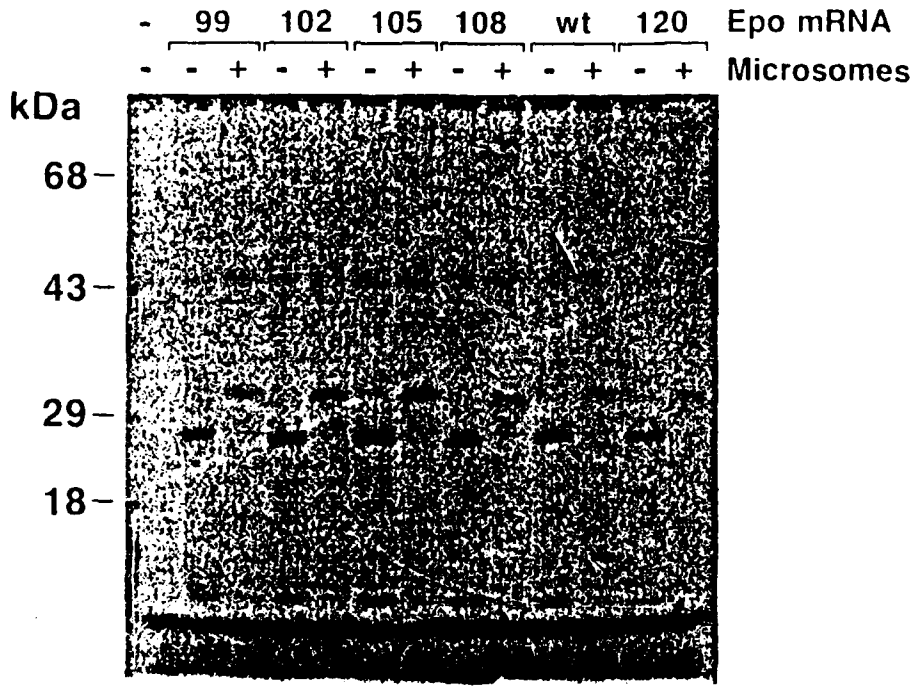
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